Differentiation of Monocyte Isolated from Leukocyte Depletion Filters into Immature Dendritic Cells Resistant to Stimulation with Lipopolysaccharide

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Abstract: Background/aim: DCs have been proposed as a tool to develop immunotherapies for cancer, chronic infections and autoimmune disease, as well as for induction of transplant tolerance; however, the rarity of DCs has hindered their use. Therefore, we aimed to generated DCs *in vitro* from leukocyte depletion filters (LDF). **Methods**: Flow cytometry, bright field microscopy, and ELISA techniques were used to determine the phenotype, morphology, and functional properties of DCs isolated from LDF, and compared to that isolated from buffy coat (BC) of the same sample. **Results**: LDF-isolated monocytes were able to differentiate into DCs with typical morphology and phenotype of immature DCs; however LDF derived DCs showed less adhesion to culture plate than cells derived from BC. Upon stimulation with lipopolysaccharide (LPS) as maturation inducer; LDF-derived DCs showed lower percentages of all maturation markers in comparison to BC-derived DCs. On the other hand these DCs had lower ability in secreting IL-12p70 and IL-10 than DCs derived from BC **Conclusions**: Our data suggest that LDF can be used as an important source for generating DCs that may replace standard BC preparations for research applications. Further researches are ongoing to establish the optimum conditions for production of DCs with full functions.
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1. Introduction

Dendritic cells (DC) are professional antigen presenting cells that play an important role in the initiation of immune responses and the induction of tolerance (Guermonprez et al., 2002; Lewis and Reizis, 2012). There is considerable interest in the generation of tolerogenic DC as a promising clinical tools for treatments of autoimmune diseases, allergies and transplant rejections (Benichou and Tocco, 2013; Frischmeyer-Guerrerio et al., 2011; Novak and Allam, 2011). Therefore it is important to select or generate the optimal tolerogenic DC type that is best suited to tolerize a specific disease (Oh et al., 2011). However, a major weakness in their application *in-vivo* is their conversion into mature-immunogenic DCs during infections or inflammation (Diou et al., 2010; Li et al., 2008). Hence, the maturation state of DCs is a critical determinant of their tolerogenic capacity. Indeed, immature DC are exceptionally sensitive to plentiful types of maturation stimuli, even vigorous pipetting (Jiang et al., 2007). Therefore, generating maturation-resistant DCs is the main goal for numerous researchers due to their importance in induction of tolerance in certain disease conditions (Berger et al., 2009; Xin et al., 2012).

Previous studies had reported that certain agents were found to induce a maturation-resistant phenotype of DC. These include, but are not limited to TGF-β plus IL-10 (Duan et al., 2005; Steinbrink et al., 1997), dexamethasone (Bosma et al., 2008) or vitamin D3 analogues (Brosbol-Ravnborg et al., 2013). These studies demonstrated that the generated immature DC, characterized by a low expression of surface MHC II molecules and low to absent levels of costimulation by CD80/CD86, led to profound inhibition of the ability of these DC to stimulate T cells (Lutz et al., 2006). Moreover, DCs play an important role in the innate immunity by secreting cytokines such as IL-12 and IL-10 that are involved in host defences (Maeda et al., 2003). In addition, DCs activate NK and NK T cells that destroy selected targets (Kassim et al., 2009, Viaud et al., 2009). DCs also exhibit significant plasticity and their main function is to stimulate either Th1 or Th2 that can be skewed by cytokine environment (Stumbles et al., 1998, Lopez et al., 2001). Therefore, the production of these cytokines, which are all indicator of DC maturation and functional ability to initiate Th1 or Th2 type immune responses is crucial to investigate. On the other hand, incomplete signals to T cells *in vivo* may be a

potential for polarization of T cells (Fogal-Petrovic et al., 2007). In many countries, leukocyte depletion filters (LDFs) used to filtrate blood units prior to storage is currently a systematic procedure followed by blood banks, besides it is used as a source for certain blood products such as red blood cell and platelet concentrates. Blood components can be either produced by using LDF of whole blood donations or by collecting it directly from the donor by aphaeresis technology. There are several potential benefits of leukocyte depletion including reduced immune complications and transfusion transmission of some cell-associated viruses (Andreu, 1991; Lane, 1994; Visconti et al., 2004). The leftover of this process, leukocyte fraction, considered a biological waste which contains viable cells including monocytes. We aim to investigate the possibility of using leukocytes depleted fraction to generate DCs in liquid culture; and to test these cells for the expression of conventional DC markers and functional ability to produce cytokines. The results will be compared with those obtained on monocytes obtained from blood of the same sample units.

2. Material and Methods

Isolation and Purification of human peripheral blood monocytes

Blood samples were obtained from healthy blood donors, after their consent and the approval of the Ethical Committee of the Centre. Peripheral Blood Mononuclear cells (PBMC) were obtained from either buffy coat (BC) or leukocytes depletion filters (LDF) provided by the Transfusions and Blood Bank Centre. Blood samples (typically 450 ml) were less than 24 hours old when processed through a LDF by gravity flow. This process takes about 30 min and it is performed at room temperature (RT). Once blood has been filtered, the storage bag containing the erythrocytes is sealed and separated, whilst the donation bag remains connected to the LDF. LDF are then maintained at 4°C until used. Cells were carefully flushed back from the LDF using 60 ml of sterile cold PBS containing 2 mM EDTA. Cell suspension was recovered into the donation bag. The volume of cell suspension recovered was about 60 ml in all samples processed. The filter was then separated from the donation bag and discarded. The cell suspension was diluted 1 to 5 with PBS containing 2mM EDTA. PBMC were obtained in both cell suspensions (LDF and BC) by standard density gradient centrifugation (400 g x 30 min. at RT) (Lymphoprep, Axis Shield, Oslo, Norway). The remaining erythrocytes were lysed with ammonium chloride RBCs lysing solution. Collected cells were washed twice in PBS containing 2 mM EDTA and further processed. Cells were counted in a haemocytometer and viability was assessed by trypan blue exclusion assay.

Generation of DCs from BC and LDF

DCs were derived from PBMCs, as described previously (Romani et al., 1996). Briefly, the mononuclear cells either from BCs or from LDF was resuspended in RPMI-1640 (Cellgro, Mediatech, and Herndon, VA) supplemented with 20 mM HEPES buffer (Cellgro, Mediatech), 100 units/ml penicillin-streptomycin, and 2 mM glutamine (Invitrogen, Grand Island, NY). Cells either from BCs or from LDF were allowed to adhere to 6 wells tissue culture plate at a concentration of $15x10^6$ cells per well. After 2 h at 37°C, non-adherent cells were removed. Plastic-adherent mononuclear cells were cultured in complete RPMI-1640 medium supplemented with 800 U/ml of recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF; Peprotech, Rocky Hill, NY; specific activity $\geq 1x10^7$ units/mg), and 500 U/ml rh IL-4 (Peprotech; specific activity $\geq 5x10^6$ units/mg). To induce maturation, Lipopolysaccharide (LPS; 1 µg/ml, Sigma Chemical Co., St. Louis, MO) was added to DCs on day 7 of culture for 48 h.

Immunophenotyping Studies

Single-colour Immunofluorescence analyses by flow cytometry were performed using the following panel of monoclonal antibodies (MoAbs): anti-human CD86; anti-CD80; anti-human HLA-DR; anti-CD14; anti-CD83; anti-CD11c. Isotype-matched relevant MoAbs were used as negative controls. Cells were incubated in the dark for 30 min at 4°C with each MoAbs. After washing, cells (10^5) were resuspended in PBS and analyzed by flow cytometer. At least 5,000 events were acquired for each condition using a FACS Calibur flow cytometer and analyzed with Summit (Dako, Colorado, USA) software. Gating on DCs population was done to exclude dead cells and contaminating lymphocytes by forward and side scatter properties. The results are shown as overlaid histograms.

Bright Field Microscopy

Differentiation of human DCs from peripheral blood monocytes or leukocyte depletion filter during seven days was examined using bright field inverted microscope (NIKON, Eclipse TE 200) equipped with digital camera (NIKON, DXM 1200). DCs were also photographed at X400 original magnification.

Assessment of IL-12 and IL-10 Production

Concentration of IL-12p70 and IL-10 released by BC and LDF human monocytes-derived DCs (10^6/ml) were measured using ELISAs employing the multiple-Ab sandwich principle (Quantikine, R&D Systems). These assays specifically detect human IL-12 p70 (the biologically active heterodimer) and human IL-10, respectively.

The IL-12 p70 ELISA specifically recognizes the IL-12 heterodimer without cross-reactivity with the individual subunits of the dimer. The minimal detection level for all these assays was 0.5 pg/ml for IL-12 p70 and 2.0 pg/ml for IL-10.

Statistical analysis

The results will be expressed as the mean ± SD of at least three different experiments. Results will be analyzed with the paired Student t-test, and *p* values < 0.05 will be considered statically significant.

3. Results

Culturing monocytes-enriched blood mononuclear cells with GM-CSF and interleukin (IL)-4, led monocytes to become non-adherent and acquired DC morphology. It was found that monocytes from LDF showed a fewer capacity to adhere to culture plates than those from BC. DCs morphology was determined during the differentiation of human blood monocyte either obtained from fresh blood BC or from LDF using bright field inverted microscope. Cells were monitored in tissue culture plate for seven days and after stimulation with LPS. As shown in figure (1-A) one day old monocytes were small in size and firmly adherent to tissue culture plate.

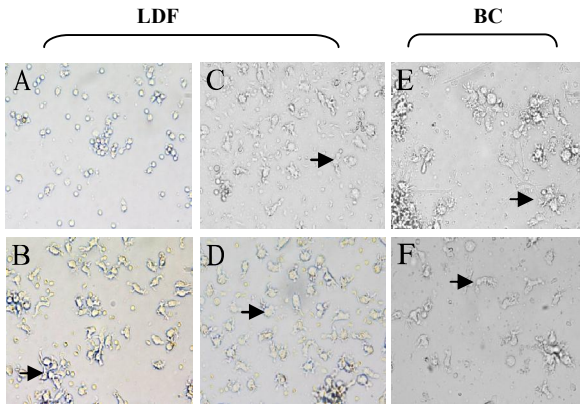

Figure 1. The morphology of monocytes obtained from either BC or LDF during differentiation into DCs. Monocytes were cultured in specific medium for 8 days and refed with GM-CSF and IL-4 cytokines twice at day 2 and 4. **A** and **B** represents cells on day 1 and 4. **C** and **D** showed unstimulated and LPS stimulated DCs on day 8 respectively obtained from LDF. **E** and **F** represent DCs on day 8 unstimulated and stimulated with LPS respectively obtained from BC. Arrows in **B** shows typical cell cluster during DCs differentiation. Arrows in **C** and **E** shows unstimulated DCs with irregular shape and dendrites. Arrow in **D** and **F** shows mature DCs with long dendrites or veils. Original magnification, X400.

On day two post seeding, the cells started to aggregate and formed clusters. The formation of clusters was also observed and increased during day four (Fig 1-B) along with the gradual detachment from the tissue culture plate. Moreover, cells changed in size and shape (Fig 1-B and D). A typical morphology of differentiated immature DCs with irregular shape and expression of cytoplasmic processes or veils was observed in figure 1-C and 1-E. On day seven all cells were stimulated with LPS or were left unstimulated then incubated for two days. After LPS-stimulation, DCs become typically mature expressing long cytoplasmic veils and seem elongated whereas unstimulated cells remained irregular with veils. The microscopic studies showed typical DCs morphology in both BC and LDF-derived DCs (**Fig 1-D and F**).

In addition, LPS stimulation to immature BC-derived DCs resulted in an increase in the expression of the co-stimulatory molecule CD80, CD86, CD11c and DC maturation marker CD83, as well as MHC class II in comparison to unstimulated DCs. Down regulation of monocyte marker CD14 was also observed. Although similar results were observed in LDF generated DCs; the expression of CD80, CD86 and HLA-DR was significantly lower than BC-generated DCs. The levels of CD83 was also slightly increased upon LPS-stimulation, however, it was lower than that of DCs generated from BC. On the other hand, CD14 was decreased in both cultures **(Table 1).**

Table 1: Percentages of BC and LDF monocytes-derived dendritic cells surface markers as determined by flow cytometry upon stimulation with LPS.

Surface Marker	BC-DCs		LDF-DCs	
	Blank	+LPS	Blank	+LPS
D14	6.0 ±1.1	**2.1 ± 0.7	0.8±0.03	0.3±0.02
CD11c	91.1 ± 7.3	97.2±4.8	83.7±3.1	82.3±3.7
CD80	81.6±5.9	97.7±6.6	51.5±6.5	*77± 4.9
CD86	36.9±7.3	**97.9±6.6	60.1±7.2	65.6±5.8
CD83	7.8±1.3	**93.5±12.7	34.8±2.1	36.7± 5
HLA-DR	90.2±5.6	99.5±7.1	79.2±7.6	83.4±5.5

(* p<0.05, ** p<0.01).

To determine the secretion of IL-12 (p 70) and IL-10; DCs generated from either BC or LDF were incubated with Lipopolysaccharide (1 µg/ml) for two days. The secretion of IL-12 and IL-10 by DCs generated from BC was significantly ($p<0.05$) elevated in response to LPS compared to unstimulated DC. However, DCs generated from LDF showed low concentrations of IL-12 and IL-10 in both unstimulated and stimulated DCs (**Figure 2 -A and 2-B**).

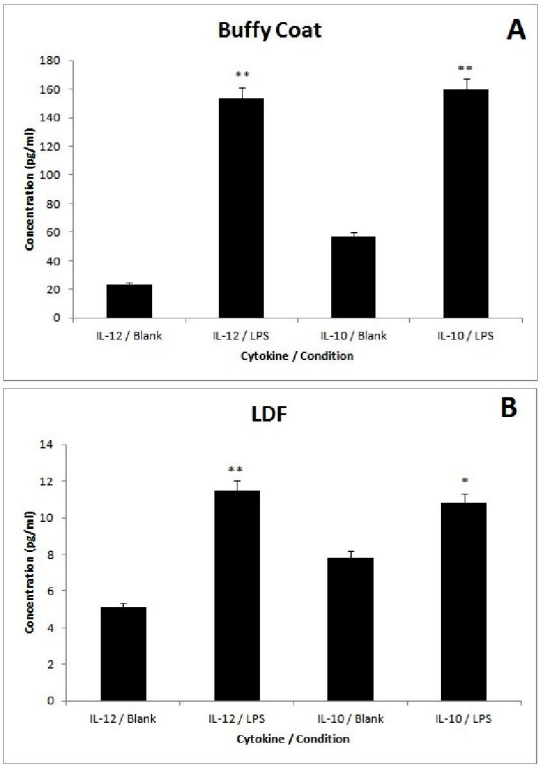

Figure 2: IL-12 and IL-10 (A) productions by human Monocyte-derived DCs obtained from BC or LDF (B) in response to LPS (1 µg ml^{-1}) stimulation. Cytokines production was determined by specific ELISA from DCs culture supernatant stimulated with LPS and unstimulated cells after 48 hours of incubation. (* $p<0.05$, ** $p<0.01$).

4. Discussions

The maturation state of DCs is vital for their tolerogenic capacity. As immature DC have been demonstrated to delay allogeneic transplantation in mice. Despite the positive effect on the graft survival, the delay of rejection was only moderate as DC maturation occurred *in vivo*, shown by CD86 upregulation (Fu et al., 1996). This study underlined the prerequisite of maturation-resistance. Therefore, generating maturation-resistant DCs is the main goal for numerous researchers due to their importance in induction of tolerance in certain disease conditions (Berger et al., 2009; Oh et al., 2011; Xin et al., 2012).

In the present study we aimed to investigate the possibility to generate DCs from monocytes separated from LDF for clinical use in a large scale. We report here that substantial numbers of monocytes separated from LDF and cultured in presence of IL-4 and GM-CSF develop with high efficiency into typical DCs, as defined by morphology and membrane phenotype. Moreover, the data obtained from our experiments using monocytes adherence method demonstrate the commitment of monocytes to differentiate into DC. However, it was found that monocytes from LDF showed a fewer capacity for adherence to culture plates than that separated from BC. It could be possible that the different buffer used to elute the cells affect this capacity as was previously reported (Bernard et al., 1998). In addition, we found that LDF-derived DCs neoexpressed a high level of the co-stimulator molecule CD86 and HLA-DR, but they lacked the CD83 marker of DC full maturation. When these cells were further stimulated with LPS, they increased the expression of all DC-specific markers except of the maturation marker CD83, however, this increase was lower in comparison with DCs generated from BC, indicating that LDF-derived DCs are in an incomplete maturation state.

DCs usually gain their immunostimulatory or immunoregulatory response depending on the type of signals they receive (Bruckner et al., 2012). Upon stimulation, immature DCs up-regulated the expression of DC specific markers including CD11c, MHC class II molecules, costimulatory molecules CD80 and CD86, and most DC maturation marker CD83. In the mean time they lost the monocytes' membrane marker CD14. These data were also reported when human DCs were generated for clinical and experimental use (Dong et al., 2011; Granucci et al., 1999; Rescigno et al., 1999; Verhasselt et al., 1997). It is worth mentioning that both BC and LDF generated DCs showed consistent trend in the expression of these maturation markers but in variable degrees, being lower in LDF-generated DCs specially the maturation marker CD83.

The ability of these DCs to secret IL-12 or IL-10 was varied between BC and LDF- derived DCs. This can be explained by the reduction or attenuation of adhesion molecules expressed on filtered leukocytes as previously reported (Chen et al., 2002; Alaoja et al., 2006). In addition, to the reduced adhesion to LPS-stimulated LDF- derived DCs. These findings may suggest the inability of LDF-derived DCs to induce either Th1 or Th2 immune responses. Moreover, our result indicated that these DCs generated from LDF may gain either Th1 or Th2 potential depending on the type of maturation stimulus or use a combination of cocktails from LPS, IFN-γ, IL-1β and PGE-2 to induce the production of either IL-12 or IL-10 as previously reported (Municio et al, 2011). This may be related to the experimental procedures of isolating monocytes from filter that been contaminated with lymphocytes such as T cells and their cytokines that may affect and suppress maturation and antigen presentation function of DCs developed as reported recently (Shimabukuro-Vornhagen et al., 2013).

Conclusions:

Our data indicated that the generation of immature DC from leukocytes depletion filter (LDF) under GMP conditions create DC resistant to maturation as defined by morphology, membrane phenotype, and lower ability to generate cytokines. This resistance could not be observed for BC-derived DC generated under the same conditions. These immature DCs may be used as tolerogenic DCs in clinical application such as transplantation rejection, autoimmune therapy, and allergy. However, further studies are necessary before this practice can be recommended.

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